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STUDIES OF WOUND HEALING IN THE PRESENCE OF AN ANGIOGENESIS FACTOR

Final Report

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Basic fibroblast growth factor (bFGF) was found to increase wound healing in a healing-impaired model; the db/db mouse. When bFGF was applied to 6 mm punch biopsy wounds, a dramatic increase in granulation tissue formation was observed. bFGF-treated wounds appeared very similar to control normal wounds in respect to dermal thickness, compactness of dermis, numbers of migratory cells and number of capillaries. No effect on the epidermis was observed. We conclude, therefore, that bFGF may be a useful modality for the treatment of wounds in healing-impaired or healing-compromised patients.

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FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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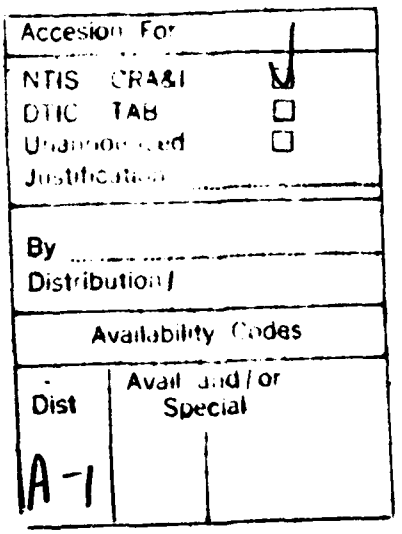

Summary

The object of this study was to ascertain whether administration of basic fibroblast growth factor (bFGF) increases wound healing in animals. To this end the effects of bFGF on incisional and punch biopsy wounds in normal mice were monitored. No increase in the rate of healing was observed. Since the rate of healing in these normal animals was extremely rapid, wound healing experiments were performed next using healing impaired animals. Streptozotocin treated mice showed no increase in the rate of healing when treated with bFGF. Diabetic mice (db/db) did show a decrease in rate of wound healing when compared to heterozygous controls. These mice had an increase in the dermal parameters of wound healing when exposed to bFGF. The effect was specific, dose dependent and was eliminated by boiling the bFGF or incubation with antibodies to bFGF. Keywords: Angiogenesis Growth Factors (AW)

Introduction

A number of studies have indicated that the healing of wounds is dependent upon the activities of specific growth factors (ten Dijka and Iwata, 1989). In addition the inclusion of antibodies to bFGF in a rat wound healing model appears to delay wound neovascularization (Broadley et al., 1989), while the application of EGF and PDGF 1989 have been reported to increase wound

repair in both humans and animals.

Because bFGF is a potent angiogenic factor and angiogenesis is believed to be critical for aspects of wound repair, the following studies were conducted to measure the effects of bFGF on wound healing in animals. bFGF was tested on both normal and healing impaired mice. Both slit wounds and punch biopsy wounds were employed. The rate of reepithelialization, granulation tissue formation, and tensile strength were measured.

The application of bFGF appeared to have significant effects on the dermal parameters of wound healing in healing impaired mice. There was little or no effect on the epithelial aspects of wound healing. No effects were observed on the healing parameters in normal mice.

Methods

Animals. - Female mutant diabetic mice, C57BL/ksJ db/db, and their littermates (db/+) were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained on a standard laboratory diet plus water ad libitum, and used at 8 weeks age. Before the experiments, mice were individually housed and checked for urinary glucose by reagent strips (Miles Laboratories Inc., Elkhart, IN). Five week old female Balb C mice were also used.

In order to induce a diabetic state with streptozotoacin, female mice were injected intraperitoneally every day for 5 days with 40 mg/Kg of streptozotacin. The mice were held for four weeks and the degree of diabetes determined by checking urinary glucose levels. Only mice which showed a high urinary glucose level were used.

Preparation of Reagents. - Recombinant bFGF was a gift from Synergen, Inc., Boulder, CO. Carboxy Methyl Cellulose (MW .250 Kd) was purchased from Polysciences Inc. (Niles, IL). The vehicle solution consisted of 1.5 % carboxy methylcellulose and 0.5% mouse serum in sterilized phosphate buffered saline solution. Different concentrations of bFGF solution were prepared by adding concentrated recombinant bFGF to the vehicle solution. Polyclonal antibodies against recombinant bFGF were raised in rabbits as described (Joseph-Silverstein et al., 1988) and IgG fractions were prepared by protein A-Sepharose column chromatography and lyophilized. Placental bFGF was prepared as described by Moscatelli et al. (1986).

Wounding. - Mice were anesthetized with sodium pentobarbitol solution (40 mg/kg, intraperitoneally) and their dorsal hair was clipped. Two full thickness round wounds were prepared vertically on the back of each mouse with

a punch biopsy instrument (6 mm diameter, George Tiemann and Co., Long Island City, NY). After the operation, 20 ul of bFGF or vehicle solution was applied. A single mouse received the same test solutions in each wound to prevent possible effects from leakage from one wound to the other. Once the wound was almost dry, the mice were allowed to recover from the anesthesia. The wounds were kept open during the experiment. In some experiments, mice were given test solutions once a day until the fifth day.

Sample Preparation and Histological Evaluation. - On the indicated day, the mice were sacrificed by cervical dislocation using care not to pull back the skin. The wounds were excised and fixed in 10% buffered formalin solution. After overnight fixation, the tissue was trimmed and cut through at the widest margin. The tissue was embedded in parafin and sectioned in 5 um increments. Six sections were placed on a slide, and stained with hematoxylin and eosin.

Histological evaluation was done in a blind way. Of the six sections on any one slide, the section with the widest original wound margin was used for scoring. Sections which indicated abscess formation were excluded from the evaluation. The parameters measured were wound closure, granulation tissue thickness, matrix density, number of infiltrated cells, and number of

capillaries. Each of the parameters was graded numerically as described below to permit average scores to be compiled for each parameter.

Wound Closure. - the degree of wound closure was measured and given a value of 0 to 10; 0 was equivalent to no closure and 10 was equivalent to complete closure by reepithelized keratinocytes.

Dermal Tissue Thickness.- A value of 1 equals a thin granulation, 2 equals moderate granulation, 3 equals a thick granulation, 4 equals a very thick granulation.

Matrix Density. - The degree of dermal matrix deposition was determined and scored as 1 equals edematous with little matrix, 2 equals a small amount of coarse matrix, 3 equals a moderate amount of matrix, 4 equals dense matrix.

Infiltrated Cells. - As an index of the degree of infiltrated cells, the number of fibroblasts and macrophages was estimated. Polymorphonuclear cells and lymphocyte were excluded from the counting. A score of 1 equals few cells, 2 equals a moderate number, 3 equals many cells, 4 equals very many cells.

Capillaries. - The number of mature capillaries was counted in the complete wound cross section at X100 magnification. A score of 0 equals 0-4 capillaries per wound, 1 equals 5-14 per wound, 2 equals 15-24 capillaries per

wound, etc.

Tensile Strength

A full thickness, vertical dorsal incision (3 cm) was made with a scalpel. After the application of bFGF or the vehicle solution, the incision was closed using a monofilament Nylon suture (5-0, Ethicon Inc., Sommerville, NJ) placed at 1 cm intervals. Mice were sacrificed on day 9 post-wounding, sutures were removed, and three strips of skin (about 1 cm wide) were taken. The first strip was used for histological evaluation. The remaining two strips were kept wet with PBS and used for fresh tensile strength measurements. A tensinometer was made using a spring balance (Maximum 250g, Ohaus Scale Corp., Florham Park, NJ). A force was applied across the incision at a constant speed (1 cm/sec). The breaking strength was the point of maximal stress before wound separation, and was expressed as g/mm incisional width. Measurements were done in a blind way.

Results

Our initial experiments indicated that linear wounds which were stapled together healed so rapidly that no effect of bFGF could be observed. Moreover, the uneven edge formed by the two opposing wound margins as well as

the debris in the wound made scoring difficult.

Therefore, early in our experiments we shifted to the use of punch biopsy wounds which provided a relatively large wound whose edges slowly converged upon each other and which could be monitored by histological techniques.

Using this approach, we monitored several parameters of the assay. These included comparing the stimulation of the vehicle, methylcellulose, to bFGF plus vehicle (Table I). The results indicated that bFGF gave only a slight increase in the number of infiltrated cells, granulation thickness, and number of blood vessels when compared to vehicle by itself. While these differences may be significant (see below), the degree of change was too small to be analyzed experimentally.

Dose-response experiments were conducted next (Table II). No clear response was observed as 4 ng bFGF gave essentially the same response as 5,000 ng. In this and several other experiments conducted during year 1, the sectioning was very poor. This was later corrected by having all samples sectioned by the Pathology Department at New York Hospital.

Since heparin is known to stabilize bFGF, we conducted an experiment in the presence of heparin. The data (not shown) indicated that there was no significant difference in the degree of wound healing with bFGF plus heparin

vs. bFGF alone.

These results, accumulated during the first year of the contract, indicated that any effect of bFGF on normal mice was very small. Additional experiments with wide incisional wounds also failed to demonstrate an effect of bFGF (data not shown). We then shifted our efforts to develop a healing impaired model of wound healing. We reasoned that an effect of bFGF might be more obvious with this model.

Our initial experiments with healing impaired animals utilized streptozotocin treated mice. These animals were severely diabetic as judged by their urinary glucose levels. However, treatment of punch biopsy wounds failed to induce an increase in any of the wound healing parameters monitored (Table III).

We then examined the effects of bFGF in an inbred strain of mice, db/db. This mouse becomes diabetic and obese as it ages. By eight weeks of age, all mice are diabetic as determined by urinary glucose levels.

As shown in Table IV, heterozygous, control db/+ littermates had good wound closure rates and granulation tissue formation. The application of bFGF to these mice did not promote a significant increase in wound healing, although it had a small effect. db/db mice had impaired wound healing compared to their heterozygous litter mates (Table IV). The differences in

the rates of wounding healing were apparent primarily in the dermal parameters measured such as granulation tissue thickness, matrix density, infiltrated cells and capillary numbers. A slight difference between the two groups in the rate of wound closure was also observed in this experiment. When bFGF (5 ug) was applied to the wounds of db/db mice, a strong response was observed in all of the dermal parameters, and a slight response occurred in the degree of wound closure. The addition of bFGF to wounds in db/db mice increased the dermal responses which made the degree of wound healing almost equal to that observed in the control heterozygous mice. The histological differences between control and diabetic mice in the presence and absence of bFGF can also be seen in Figs. 1 and 2. A large increase in the amount of granulation tissue after bFGF application is easily visible.

The dose of bFGF required to stimulate increased healing in db/db mice was next determined. Doses of 0.05, 0.5, and 5 ug were applied once a day for five days to wounds (Table V). At 0.05 ug/day of bFGF, a small increase was seen in the number of infiltrated cells as well as the number of capillaries. At 0.5 ug/day of bFGF, strong increases in all of the dermal parameters were apparent. The number of capillaries was significantly higher than that seen in mice receiving the lower dose. At 5 ug/day of bFG,F a further increase was observed. Thus, the effective dose of bFGF required for a significant

increase in wound healing is between 0.05 and 0.5 ug/day when applied multiple times. However, none of the doses tested had an effect on the degree of wound closure.

Since Davidson et al. reported that iodinated cartilage-derived growth factor, which appears to be a form of bFGF, disappeared from wounds within 24 hr after injection, we next tested whether significant differences in healing occurred between single vs multiple applications of growth factor. Single dosing at 0.5 ug, as well as 5 ug, gave similar responses as multiple doses at 5 ug (Table VI).

The preparation of bFGF in methylcellulose might increase the amount of growth factor retained at the local site because of its increased viscosity. However, application of bFGF in PBS generated the same response as bFGF in methylcellulose solution (Table VI). To insure that the effects measured resulted from the bFGF, two additional experiments were conducted. Boiled bFGF (5 ug/day) did not significantly increase the number of infiltrated cells or capillaries (Table VI). Furthermore, bFGF pretreated with anti-bFGF IgG, which neutralized the effect of bFGF in vitro on bovine capillary endothelial cells, blocked the histological responses, but non-immune IgG did not (Table VI). These data clearly showed that bFGF is responsible for the observed responses.

In order to measure the effects of bFGF on wound healing in db/db mice as a function of time, we quantitated the effects of the growth factor at 5, 8, 12 and 18 days post-wounding. These results were compared to the values in non-treated db/db wounds (Table VII). All of the parameters monitored in the bFGF-treated mice exceeded those in non-treated mice. However, significant differences were not observed in the rate of wound closure. All the granulation parameters, especially infitrated cell number and capillary number, showed significantly higher scores in bFGF-treated mice ($p < 0.01$). All of the granulation parameters in bFGF-treated mice continued to increase between 8 and 12 days. Interestingly, between 12 and 18 days, the granulation response appeared to begin to resolve. There was a decrease in the thickness of the granulation tissue, the number of infiltrated cells, and the number of capillaries. Meanwhile, matrix density continued to increase, which suggests matrix maturation and remodeling.

We next attempted to measure breaking strength in incisional wound to confirm that increased matrix formation contributed to wound strength. Figure 3 illustrates the results of tensile strength measurements in db/db mice and db/+ wound preparations. Healed wounds from db/db mice had only 56% of the tensile strength observed in healed wounds from db/+ litter mates.

Application of 5 ug bFGF in a single dose increased the strength of the

wound 24% in normal littermates, and 46% in db/db mice. As a result, tensile strength in bFGF treated db/db wounds reached a level similar to that of wounds from non-treated heterozygous littermates. These data suggest that increased granulation tissue contributes to the strength of the wound site.

Conclusions

These experiments provide evidence that recombinant bFGF is capable of significantly improving the degree of dermal healing in healing impaired mice. The observed increases in granulation tissue thickness, infiltrated cells, capillaries, and tensile strength are consistent with the proposal that bFGF stimulates the granulation response. Basic FGF appeared to have little effect on the rate of healing in healthy animals.

Unlike EGF, bFGF appeared to have little effect on the rate of wound closure. Since keratinolytes respond to bFGF (O'Keefe et al., 1988), this result was somewhat surprising. Perhaps, because we used an open wound system, the presence of the large crust on the granulation tissue affected the migration of keratinocytes. Therefore, it will be of interest to test the effect of bFGF on wounds with occlusive dressings.

It is also interesting that only a small effect of bFGF on the rate or degree of healing was observed in normal mice, though there are several

reports which demonstrated positive effects in normal rats (Davidson et al., 1985, McGee et al., 1988). Normal mice may have sufficient amounts of growth factors and a normal wound healing system so that excess growth factor has no effect.

The effective dose of bFGF required for a significant increase in wound healing was determined to be 0.5 ug/day after multiple applications. A single dose of bFGF at 0.5 ug was also effective. The reason why a single dose was as effective as mutiple doses is not clear. Multiple doses of high concentrations of bFGF did not induce unlimited granulation tissue formation, and after wound closure, a decrease of granulation tissue and induction of matrix occurred suggesting that bFGF can be used as a self-limited, wound healing potentiating agent.

The effect we have found in these studies may be applicable to the healing of wounds other than those found in healthy individuals. This may include dicubitus ulcers, diabetic ulcers and well as wounds in patients who are malnourished, hypothermic or undergoing treatment with other agents known to affect wound healing.

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Table I

Wound Healing in Normal Balb C Mice - Effect of bFGF

Parameter

Day	Mice	Wound Closure	Epidermal Thickness	Granuloma Thickness	Infiltrated Cells	Degree of Vascularization
5	C	0	0	1.5	1.7	1.7
	FGF	0.5	0.5	1.7	2.5	1.7
8	C	0.6	1.3	1.6	2.0	2.3
	FGF	0.75	1.2	3.0	3.0	3.0
11	C	1.0	2.0	1.5	1.7	1.7
	FGF	1.0	1.7	2.0	2.2	2.2

C = Control Mice - Methylcellulose only.

FGF = Experimental Mice - bFGF + Methylcellulose

Table II

Effects of Different Doses of bFGF on Wound Healing in Balb C Mice

Parameter

Sample	Wound Closure	Epidermal Thickness	Granuloma Thickness	Infiltrated Cells	Degree of Vascularization
PBS	0.6	1.1	1.1	2.0	1.1
Methyl-Cellulose	0.8	1.1	1.5	2.2	1.5
FGF (5 ng)	0.7	1.0	1.8	2.4	1.7
FGF (500 ng)	0.8	0.8	1.6	2.0	1.8
FGF (5,000 ng)	0.60	0.8	1.7	2.4	1.8

Table II

Effects of Different Doses of bFGF on Wound Healing in Balb C Mice

Parameter

Sample	Wound Closure	Epidermal Thickness	Granuloma Thickness	Infiltrated Cells	Degree of Vascularization
PBS	0.6	1.1	1.1	2.0	1.1
Methyl-Cellulose	0.8	1.1	1.5	2.2	1.5
FGF (5 ng)	0.7	1.0	1.8	2.4	1.7
FGF (500 ng)	0.8	0.8	1.6	2.0	1.8
FGF (5,000 ng)	0.60	0.8	1.7	2.4	1.8

Table III

Wound Healing in Streptozotacin-Treated Mice

ng/day (bFGF)	Number of Mice	Percentage of Wound Closure	Epidermal Thickness	Dermis Thickness	Compactness of Dermis	Infiltrated Cell Numbers	Capillary Numbers
0	7	5.4 ± 1.5	0.4 ± 0.3	2.2 ± 0.3	1.9 ± 0.2	2.0 ± 0.3	2.0 ± 0.3
0.5	6	6.5 ± 1.1	0.5 ± 0.3	1.8 ± 0.2	1.8 ± 0.4	1.5 ± 0.2	1.3 ± 0.2
50	6	4.5 ± 1.0	0	2.3 ± 0.3	1.7 ± 0.2	2.0 ± 0.4	2.0 ± 0.2
5000	5	7.4 ± 0.5	0	2.2 ± 0.2	2.0 ± 0.3	2.2 ± 0.2	1.8 ± 0.3

TABLE IV A

Effects of bFGF on Wound Healing in db/db Mice and Normal Littermates

Treatment	N	Wound Closure	Granulation Tissue	Matrix Density	Infiltrated Cells	Capillary Number
Normal Mice (db/+) 0 ug x 5 days	10	8.8 ± 0.6	3.3 ± 0.1	3.3 ± 0.3	3.2 ± 0.2	8.1 ± 0.8
Normal Mice (db/+) 5 ug x 5 days	9	8.6 ± 0.7	3.6 ± 0.2	3.4 ± 0.3	3.8 ± 0.1	10.6 ± 1.3
db/db Mice, 0 ug x 5 days	10	7.1 ± 0.7	1.4 ± 0.2	2.1 ± 0.4	1.7 ± 0.1	2.4 ± 0.4
db/db Mice 5 ug x 5 days	10	8.4 ± 0.6	2.8 ± 0.2	3.1 ± 0.2	2.8 ± 0.2	8.2 ± 0.9

Samples were taken at 8 days post-wounding. Vehicle solutions plus and minus bFGF were applied each day for five days beginning with the day of wounding.

TABLE IV B

Dose Effect on Wound Healing in Normal and db/db Mice

A. db/+ mice

Sample	Percentage of Wound Closure	Epidermal Thickness	Dermal Thickness	Compactness of Dermis	Infiltrated Cells	Capillary Number
0 ng X 5 days (11)	6.9 ± 0.8	0.7 ± 0.4	2.6 ± 0.2	3.5 ± 0.3	3.1 ± 0.2	8.8 ± 1.2
50 ng X 5 days (10)	7.8 ± 0.9	1.2 ± 0.3	2.4 ± 0.2	3.2 ± 0.2	2.9 ± 0.2	7.2 ± 1.1
500 ng X 5 days (8)	7.0 ± 0.9	0.8 ± 0.3	3.3 ± 0.3	3.3 ± 0.3	3.4 ± 0.2	8.4 ± 1.6
5000 ng X 5 days (8)	9.6 ± 0.4	2.3 ± 0.3	3.1 ± 0.3	3.5 ± 0.2	3.1 ± 0.3	6.9 ± 1.3

TABLE V

Dose Effect on Wound Healing in db/db Mice

B. db/db Mice

Treatment	N	Percentage of Wound Closure	Granulation Tissue Thickness	Matrix Density	Infiltrated Cells	Capillary Number
0 ug x 5 days	10	5.0 ± 0.6	1.6 ± 0.2	1.8 ± 0.2	1.8 ± 0.2	4.2 ± 1.0
0.05 ug x 5 days	10	5.0 ± 0.7	1.7 ± 0.1	1.9 ± 0.2	2.6 ± 0.3	6.8 ± 1.2
0.5 ug x 5 days	5	5.6 ± 1.1	2.8 ± 0.2	2.2 ± 0.3	3.0 ± 0	12.8 ± 1.4
5 ug x 5 days (8)	8	4.3 ± 0.9	3.0 ± 0.3	3.6 ± 0.2	3.8 ± 0.2	13.8 ± 2.9

Samples were taken at 8 days post-wounding. Solutions were applied once a day each day for five days beginning with the day of wounding.

TABLE VI

Effects of bFGF on Wound Healing in (db/db) Mice

Treatment	N	Wound Closure	Granulation Tissue Thickness	Matrix Density	Infiltrated Cells	Capillary Number
0.05 ug x 1 day	8	7.5 ± 0.8	1.6 ± 0.2	1.9 ± 0.2	1.9 ± 0.3	7.5 ± 1.5
0.5 ug x 1 day	10	6.4 ± 0.7	2.5 ± 0.2	2.5 ± 0.2	2.5 ± 0.2	12.1 ± 1.1
5 ug in MC x 1 day	8	8.9 ± 0.5	2.8 ± 0.2	2.8 ± 0.2	2.9 ± 0.1	13.5 ± 1.3
0 ug x 5 days	9	6.1 ± 0.9	1.9 ± 0.1	2.1 ± 0.3	1.8 ± 0.1	5.2 ± 0.6
5 ug in MC x 5 days	10	5.3 ± 0.7	2.5 ± 0.2	2.2 ± 0.2	2.6 ± 0.2	12.9 ± 1.8
5 ug in PBS x 5 days	8	6.3 ± 0.8	2.5 ± 0.2	2.6 ± 0.2	2.6 ± 0.2	10.1 ± 1.0
Boiled 5 ug x 5 days	9	6.2 ± 0.6	1.6 ± 0.2	2.0 ± 0.2	2.0 ± 0.2	7.1 ± 0.9
0.5 ug + anti-bFGF IgG x 1 day	7	7.0 ± 1.0	2.1 ± 0.1	2.0 ± 0	2.1 ± 0.1	7.9 ± 1.6
0.5 ug + non-immune IgG x 1 day	7	6.7 ± 0.9	2.9 ± 0.1	2.9 ± 0.2	2.9 ± 0.2	14.3 ± 2.1

Samples taken at 8 days post-wounding.

TABLE VII

Time Course of Wound Healing in db/db Mice With and Without bFGF.

Treatment	N	Wound Closure	Granulation Tissue Thickness	Matrix Density	Infiltrated Cells	Capillary Number
0 ug x 5 days	10	1.7 ± 0.2	1.3 ± 0.1	1.0 ± 0	1.3 ± 0.1	1.8 ± 0.6
8 days	9	3.6 ± 0.6	1.2 ± 0.1	1.1 ± 0.1	1.4 ± 0.2	3.4 ± 1.3
12 days	9	7.7 ± 0.6	2.0 ± 0.2	2.3 ± 0.2	2.1 ± 0.2	5.8 ± 0.8
18 days	11	10.0 ± 0	1.7 ± 0.2	3.0 ± 0.2	2.0 ± 0.2	7.0 ± 0.9
5 ug x 5 days	10	2.0 ± 0.2	1.3 ± 0.1	1.0 ± 0	1.4 ± 0.2	3.4 ± 1.1
8 days	8	3.8 ± 0.7	2.6 ± 0.2	2.1 ± 0.2	2.6 ± 0.2	11.9 ± 2.7
12 days	10	7.9 ± 0.9	2.9 ± 0.3	3.2 ± 0.2	3.2 ± 0.2	16.8 ± 1.9
18 days	10	10.0 ± 0	3.3 ± 0.1	3.6 ± 0.2	2.5 ± 0.2	10.4 ± 0.9

Figure 1. Effects of bFGF on wound repair in db/+ Mice.
Wounds were made and treated as described in Methods. Column 1 wounds which received control vehicle only; column 2, wounds which received bFGF (5 ug) plus vehicle. A, low power magnification; b, high power magnification. Samples prepared on day 8. Arrowheads delineate original wound boundary.

Figure 2. Effects of bFGF on Wound Repair in db/db Mice.
Wounds were made and treated as descsribed in Methods. Column 1, wounds which received control vehicle only; column 2, wound which received bFGF (5 ug) plus vehicle. a, low power magnification; b, high power magnification. Samples taken on day 8. Arrowheads delineate original wound boundary.

Figure 3. Tensile strength of normal and db/db wounds. Wounds were made in db/db mice and heterozygous littermates as described in Methods. Animals were treated with 5 ug/wound of bFGF or used as controls. The wounds were then prepared and tested as described in Methods. 1, db/+ control; 2, db/+ plus 5 ug bFGF; 3, db/db, control; 4, db/db plus 5 ug bFGF.

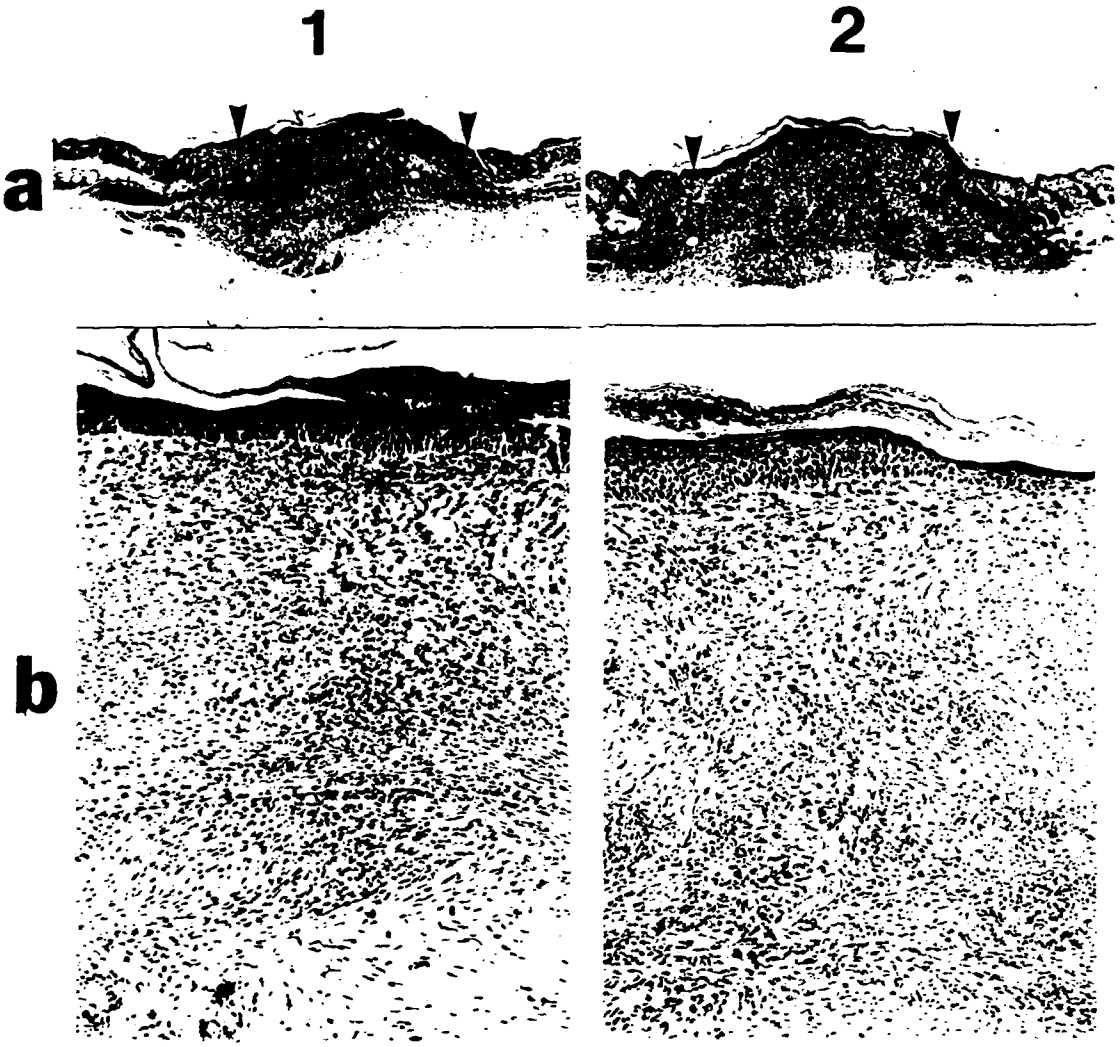

FIGURE 1

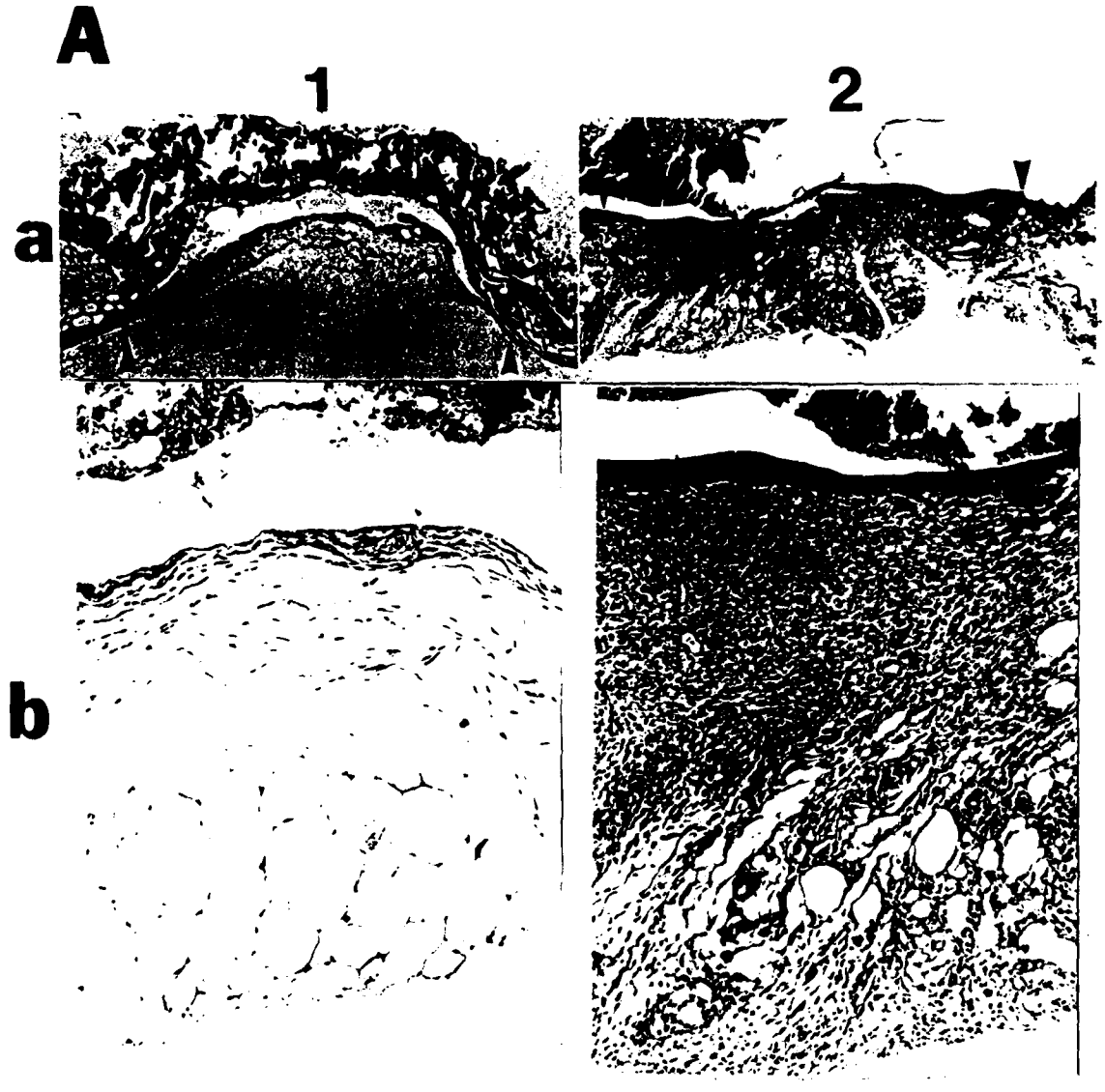

Figure 2

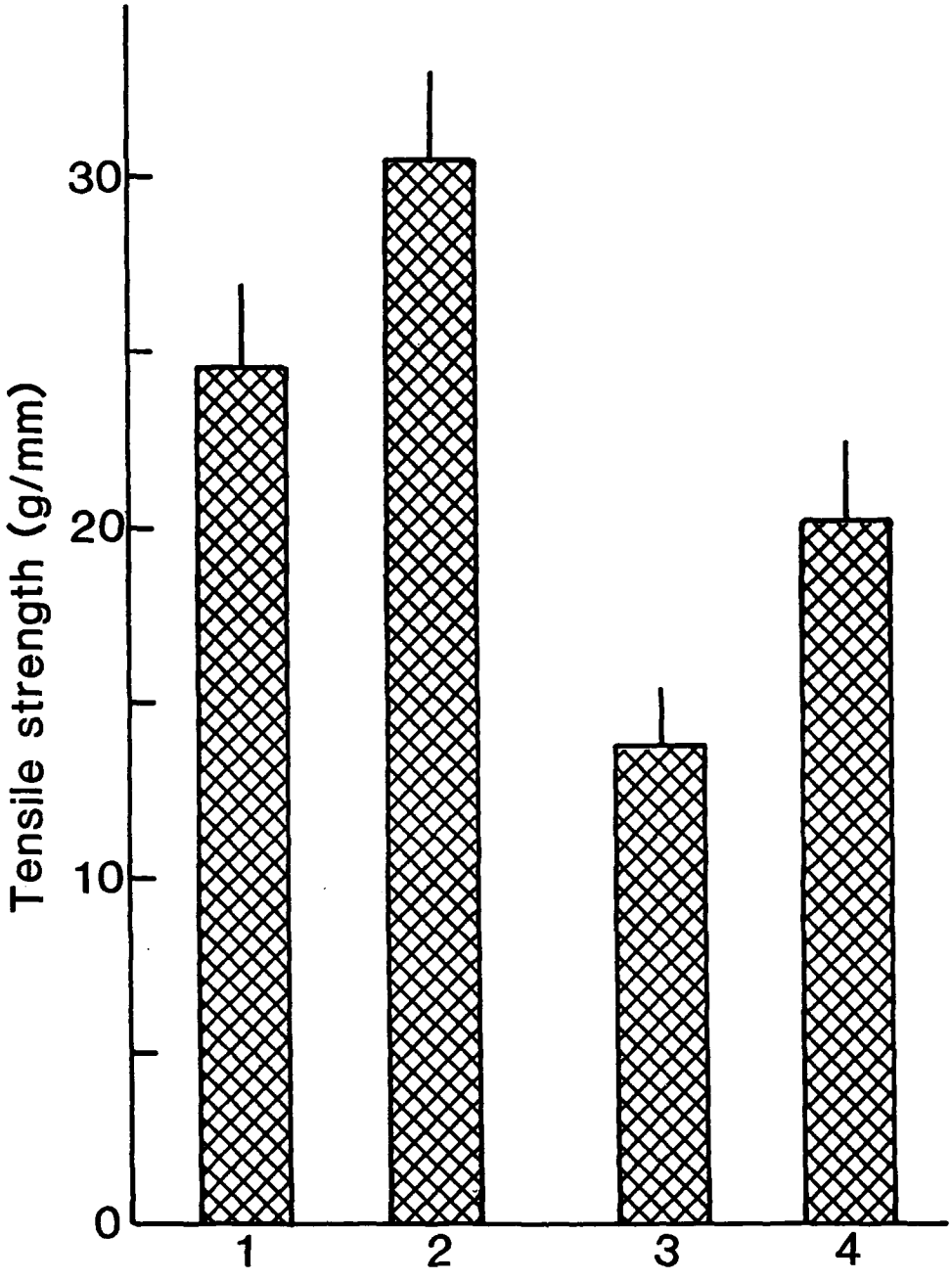

FIGURE 3